# Vaccine and antibody production in plants: developments and computational tools

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**Abstract**

Plants as bioreactors have been widely used to express efficient vaccine antigens against viral, bacterial and protozoan infections. To date, many different plant-based expression systems have been analyzed, with a growing preference towards transient expression systems. Antibody expression in diverse plant species for therapeutic applications is well known and this review provides an overview of various aspects of plant based biopharmaceutical production. Here, we highlight conventional and gene expression technologies in plants along with some illustrative examples. In addition, the portfolio of products that are being produced and how they relate to the success of this field are discussed. Stable and transient gene expression in plants, agrofiltration, and virus infection vectors are also reviewed. Further, the present report draws attention to antibody epitope prediction using computational tools, one of the crucial steps of vaccine design. Finally, regulatory issues, biosafety and public perception of this technology are also discussed.

**Keywords:** Epitope prediction; Computational tools; Genetic engineering; Plantibody; Edible vaccine.

## 1. Introduction

Currently, many types of transgenic cells are the used as source material in vaccine production. When antigens are expressed in wild or engineered systems, processing to remove toxins and host proteins, contributes to increase production costs [1]. Moreover, these systems are also sensitive to contamination by other microorganisms that often escape detection in pure vaccines. In response to the challenges faced by current pharmaceutical production practices, plant

molecular farming (PMF) has emerged as a viable technology for the production of recombinant proteins like enzymes, monoclonal antibodies (mAbs) and antigens for human and animal vaccines [2,3,4]. Plant expression systems have several intrinsic advantages vis-á-vis bacteria, yeast, insect or mammalian systems in terms of speed, costs, scalability and safety [5, 6]. In addition, plants have the capacity to perform post-translational changes which are important in protein folding, trafficking, stability and biological activity [7,8]. Plant derived antibodies (PDAbs) have been developed for the protection against different pathogens, including viruses, bacteria and fungi [9, 10]. Investigators have also analyzed plant-based expression systems with a growing preference towards transient expression systems [1,11]. There are several notable reports on stable transformation of plant nuclear and plastid genomes [12, 13, 14]. Furthermore, alternative approaches such as systems biology and gene editing have been reported for developing therapeutic products in other organisms. These studies pave the way to adopt these concepts to plant derived antibody production [15, 16].

In this article we review conventional and state of the art plant-based gene expression technologies along with some illustrative examples (Figure 1).

## 2. The expression of antigens in plants

### 2.1 Stable and transient expression of antigens

During stable transformation, the foreign gene is integrated into the genome of the cell using *Agrobacterium* or biolistic approaches (microprojection, bombardment and protoplast transformation). However, this type of transformation has some shortcomings; *Agrobacterium* has target limitations and expression of desired protein with respect to total soluble plant protein

is low (0.01% to 0.30%) [17]. Alternatively, in transient systems, the foreign gene is expressed without being integrated into the genome [17]. Traditionally, the process of generation of stable transgenic lines is time consuming and cumbersome whereas, transient expression systems are rapid and simple.

For rapid development of vaccines, attempts were made to explore transient gene expression. Examples of the transformation of antigen genes into plant genomes are outlined in Tables 1-3.

***Insert Table 1, Table 2, Table 3***

Initial studies described the production of a surface protein (spa A) from *Streptococcus mutans* (0.02 % of total leaf protein) in tobacco plant [18]. Early attempts for vaccine production used transgenic plants for expression of LT-B (heat labile) protein (0.05% of total soluble proteins) of *E. coli* in tobacco plant [19] (Rosales-Mendoza et al., 2011). In case of diarrhea, the enterotoxin B subunit (LTB) from *E. coli* and cholero toxin (CT), were expressed in chloroplast genome of tobacco, ,maize and rice [20,21,22].

**2.2 Plant virus fusion proteins**

Plant viral vectors represent a promising approach for the expression of antigenic epitopes with self-assembling viral peptides since they can mount simple and rapid infections, allowing production of large amounts of recombinant protein in susceptible host [23]. The widely-used technique for transient transformation uses recombinant plant viruses as protein-expression vectors such as potato virus X (PVX), cowpea mosaic virus, tobacco mosaic virus (TMV), and

cucumber mosaic virus (CMV) etc. A generalized diagram of recombinant PDAbs production versus selective breeding is presented in Figure 2. This explains how these specified methods can be pivotal towards effective PDAbs production. The CT of *V. cholerae* has two components, CT-A and CT-B, where CT-B is accounted for mucosal and serum immunity [24]. The genes for ctB were cloned in plant expression vectors and transformed into tobacco plants [25].

It is also feasible to express foreign proteins with viral coat protein (CP). Advantages of this "overcoat" approach are the relative ease with modified viral particles can be purified from infected tissues and the presentation of numerous copies of an antigenic peptide on the surface of a macromolecule carrier can drastically augment its immunogenicity [26]. The epitopes from various pathogens have been expressed in plants using various coat proteins from plant viruses as carriers (Table 3). The D2 epitope of the fibronectin binding protein B of *Staphylococcus aureus* was expressed with the coat protein of Cowpea Mosaic Virus (CPMV). Treatment induced both serum and mucosal antibodies against the D2 peptide. Similarly, a capsid protein from Norwalk virus was expressed in tomato [27]. In CPMV, an icosahedral viral particle with several useful properties, a coat protein gene was used to encode fusion recombinant antigens [28]. One more study reported the expression of recombinant CPMV coat protein fused with the VPI epitope of human rhinovirus in cowpea leaves [29]. One example reported the expression of Norwalk virus capsid protein (NVCP) with a 35S CaMV promoter or a potato specific patatin promoter in conjunction with *A. tumefaciens*, The construct was transformed into the tobacco and microtubers of potato to produce NVCP protein [30]. Furthermore, expression of the surface antigen hepatitis B virus (HbaAg) was reported in tobacco [31], in leaves of potato [32], in tomato [33]. In a recent study, investigators demonstrated that transformation of the *Nicotiana*

*benthamiana* with RhoA peptide resulted in successful protein expression and reduced growth of respiratory synctial virus [34].

Nevertheless, CP fusions are not tolerated as they can obstruct particle assembly and spread [35]. This has been largely overcome by introducing the foot-and-mouth disease virus (FMDV) 2A peptide between the heterologous sequence and the CP ORF (Figure 3). The expressed proteins were efficiently assembled into particles and encapsidated the viral RNA without any significant effect on the virus viability [35]. Having established the utility of the approach, several antigen candidates have reported rotavirus inner capsid protein (VP6) [36], swine fever virus (SFV) E2 glycoprotein [37], tuberculosis ESAT-6 protein [38], and the hepatitis C virus (HCV) envelope protein R9 [39]. PVX virions carrying the CSFV glycoprotein epitope produced an immunoprotective response in rabbits [37] and the R9 epitope on the surface of PVX particles was highly immunogenic in mice [39]. Other pox viruses with a variety of hosts have also been utilized to express a foreign gene as a fusion protein with CP, separated by the FMDV 2A peptide: pepino mosaic virus (PepMV) infecting *N. benthamiana* plants [40] and *Plantago asiatica* mosaic virus (PIAMV) infecting *N. bethamiana* and *Arabidopsis thaliana* [41]. The bipartite CPMV has been extensively used as a virus vector system based on transgenic for the expression of proteins in plants. The use of 2A instead of viral cleavage motifs diminished the number of virus-derived amino acids linked to the expressed foreign protein, and as in PVX "overcoat" vectors, some of the protein is present in virus particles. This approach has subsequently been used to express hepatitis B virus (HBV) nucleocapsid protein (HBcAg) [42] and small immune proteins (SIPs) specific for transmissible gastroenteritis virus [43]. In soybean-infecting bean pod mottle virus (BPMV) vectors, non-identical FMDV-2A peptide sequences were used both to assist simultaneous co-expression of two dissimilar proteins, and to

reduce instability of the introduced genetic elements [44]. Recently expression of synthetically stabilized virus-like particle (sVLP) for vaccine with D antigenicity of poliovirus was reported in *N. benthamiana* [45].

**2.3 Edible vaccines**

In developing countries vaccine costs can be a limiting factor in healthcare. A published report in Decade of Vaccines (DoV), indicated that the total cost of vaccination had increased significantly from $1.37 in 2001 (to vaccinate against six diseases) to $38 in 2011 (for 11 diseases) [46]. In another study it was reported that bacterial and viral antigens were expressed in edible plants and ingestion of these plants containing vaccine proteins has shown antigenicity. Thus, alternative economical vaccination methods such as edible vaccines have recently gained attention [47-49]. These vaccines, defined as mucosal-targeted vaccines, (which originate) bring about a prompt systematic and mucosal immune response. The functional site of mucosa-associated lymphoid tissues (MALT) is associated with the gut, lungs and bronchial system. The secretory antibody IgA functions to prevent virus entry and (also) clump antigens which are removed from the body in the mucus or faeces. It is pertinent to mention that oral vaccine supposedly sustain activity in the intestine at acidic pH [50].

The Tobacco *(N. tabacum)* plant has shown to be an efficient system for expressing different forms of antibodies, secretory IgG and IgA, single-chain variable fragments (scFv), [51], Fab fragments, and bispecific antibodies. For example, monoclonal antibodies of a single chain variable fragment (scFV) against the West Nile virus have been made as a fusion protein in glycol-engineered *N. benthamiana* [52, 53]. Inoculation with full-length or deleted versions of RNA-2 comprising the heavy and light chains of a murine blood group-typing antibody fused with the C-terminus of the small coat protein domain *via* F2A resulted in the expression of

assembled full-size IgG in *N. bethamiana* [54]. This approach has also been used to express derivatives of scFvs specific for transmissible gastroenteritis virus (TGEV) in cowpea plants and the crude plant extracts supplied *ad libitum* to neonatal pigs to provide *in vivo* protection against challenge with this enteric pathogen [43]. Further, transient expression of antigenic epitopes of the rabies virus (RV) was also reported in tobacco, spinach and *Nicotiana benthamiana*, and successful immunization of mice was observed by feeding transgenic spinach [14]. Another interesting study reported the fusion of complementary DNAs (cDNA) of cholera toxin (CT) subunit B and A2 with the enterotoxin gene of rotavirus and enterotoxigenic fimbrial antigen gene of *E. coli*. This was transferred into potato resulting in the expression of fusion antigens in transformed tissues.

These antigens were assembled into cholera holotoxin-like structures and exhibited affinity for enterocyte. The oral immunization into mice caused production of antibodies for pathogen antigens. Thus it may be suggested that edible vaccine can provide simultaneous protection against viral and bacterial pathogen [55].

There are reports about corn based vaccines and ginger derived nanoparticles, including the use of plant glycans [56-58]. In one more study, transgenic tobacco leaves and potato tubers were established which were capable of producing GAD (glutamic acid decarboxylase), a diabetes-associated antigen for treatment of Type II diabetes. The expression was detected at a level of 0.33 to 1.0 mg/g of fresh weight. Feeding 3 g transgenic plant tissue daily from 5 weeks to 8 months showed diabetes only in 2 out of 12 mice [59].

## 3. Alternative approaches for plant-derived antibodies

### 3.1 Chloroplast transformation for vaccine production

Chloroplast transformation has been widely studied for the expression of a variety of compounds [60] and the cheap production of vaccines. Chloroplast genetic engineering offers several advantages over nuclear transformation: (as) increased biosafety related to release of integrated genes into the environment due to maternal inheritance of chloroplast genomes, multiple copies of chloroplast genome per cell enables high levels of protein expression and possibility of coexpression of multiple genes provides a way to produce vaccines requiring multiple epitopes for activity, homologus recombination for transfer of transgene into chloroplast genome [61-65]. Chloroplast transformation also enables post-translational changes in expressed proteins and correct folding required for antigenicity [66]. Further, foreign protein in the chloroplast does not interfere with growth and morphology of the host plant [64]. Investigators have reported expression of many antigenic epitopes in this regard including hepatitis, cervical cancer, AIDS, and small pox. Other examples include; HPV-16 L1 VLPs epitope in tobacoo [67], Potato virus X (PVX) coat protein (CP) fused with HPV-16 E7 oncoprotein (E7-CP) in tobacoo [68], Hepatitis E virus (HEV E2) in tobacco [69], Rotavirus (VP6) in tobacco [70], HIV (p24-Nef) in tobacco, tomato [71], CTB fused with fibronectin-binding domain (D2) of *S. aureus* (CTB-D2) in *Chlamydomonas* [72], and Cholera toxin B–proinsulin fusion protein (CTB-Pins) in tobacco and lettuce [73]. Though transplastomic expression of vaccine antigens is considered relatively safe compared to nuclear expression, these are also associated with politropic effects [74] such as male sterility [75] and stunted growth of the host plant. Still, many other regulatory issues should be challenged such as, degradation of proteins, efficacy of vaccines produced in chloroplast etc.

In most of the cases, transplastomic study has been conducted in tobacco plants and other species need to be explored [76].

### 3.2 Genome editing

Though genome editing techniques are associated with manipulating DNA at a specific location or repair of existing DNA. For this purpose commonly explored technologies are zinc-finger nucleases (ZFNs), CRISPR-associated protein 9, and transcription activator-like effector nucleases (TALENs) [77, 78]. Recent development in genome editing techniques have enabled researchers to manipulate the genome at the sequence specific level for variety of purposes [78-80]. Genome editing has been used to engineer cell lines and organisms through gene knockout, deletion, correction, inversion [81-83] and chromosomal translocation [84]. Moreover, these sequence specific nucleases have been effectively used for plant genetic engineering [85]; wheat [86], soybean [87], barley, *Brassica oleracea* [88], tomato genome [89] and *Arabidopsis* [90]. Though genome editing may be caused by mutagenesis during cell culture but such reports in the generation of transgenic plants for edible vaccines are limited. One example is the production of an edible vaccine against cedar pollen allergy in rice [91] where researchres have suggested cell culture steps as cause of mutation on the basis of whole genome sequencing between transgenic line and host.

### 3.3 Epitope prediction for vaccine development using computational tools

Antibody epitope prediction is crucial step of vaccine design. Various considerations such as hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains have to be correlated with the location of continuous epitopes. Thus epitope prediction is important when designing vaccines. System level understanding of cells

with the help of *in silico* methods can also help the prediction and localization of epitopes [15]. Still, these techniques are not used for design of antigen for plantibodies but these methods are quick, accurate and can be helpful in plantibody manufacturing and may be vital in the biopharmaceutical industries (Table 4).

***Insert Table 4***

Moreover, Figure 4 also depicts the various bioinformatics based methods which can be used for epitope prediction. Some important methods are like; Artificial Neural Network [92-94], Hidden Markov Models (HMMs) and Molecular dynamics simulations (MD) [95]. Structure activity relationships (SARs), support vector machines (SVMs) and virtual screening (VS).

## 4. Common problems associated with plantibodies

There are certain important issues which needs to be discuseed towards effectual plantibodies development. Some of them are explained in this section.

### 4.1 Immuno-modulation

Immune-modulation has been used to study antigen/epitope function as well as antigen mistargeting [96]. There are certain ways in which antibody affects protein: competitive and allosteric inhibition and interference in the protein folding. Antigen mistargeting in tobacco plants resulted in scFv fragment accumulation in the ER and binding of abscisic acid (ABA). TMV infection in tobacco plants resulted in the expression of a TMV virion binding scFv fragment [97,98]. Lessening of necrotic local lesion numbers was observed during virus infection as well as increased scFv targeting to the apoplast. In another study, camelid nanobodies have been recognized as an effective countermeasure against Broad Bean Mottle Virus (BBMV)

infection. It was observed that these nanobodies controlled growth of BBMV virus in *Vicia faba* [99].

### 4.2 Development and commercialization issues

Although plants are being exploited successfully for expression of antigenic epitopes in the production of edible vaccines, there are still many challenges limiting their success. Despite the fact many vaccines have completed phase III of clinical trials [2], to date, no vaccine is available for market consumption [17]. Problems include: Improper gene transfer techniques, low levels of expression, difficulty in promoter identification, variation, codon biasing and scantly regulatory sites, inappropriate polyadenylation and unsuitable nuclear transport, mRNA instability and positional effects, epigenetic silencing, insufficient immune response after administration, regulatory issues and maintenance of GMP standard [100-102], selection of antigen and host [103] and consistency of dose [104].

## 5. Conclusions and future prospects

The present review has demonstrated several examples on the production of recombinant proteins in plants, specifically vaccine production. Nevertheless, commercialization of PDAs faces several limitations. Biosafety and environmental issues are affecting the promotion of bulk cultivation of engineered plants, however existing regulatory norms are to be followed as per guidelines as per stakeholder's country. Researchers need to increase levels of antigen expression in plants along with targeted expression but it needs more focused approach. Nevertheless, there is a requirement for further comprehensive studies of specific antigens previous to plant-based vaccines can be used as a product. Presently, USDA and FDA regulate

production of plant derived vaccines. Although these regulatory bodies are controlling the production of these vaccines, we need to deliberate upon the current insights on the evaluation of such products towards commercialization.

**Conflicts of Interest**

There is no conflict of interest among the authors.

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**References**

1. Rybicki EP. Plant-based vaccines against viruses. Virol J 2014;11:205
2. Faye L, Gomord V. Success stories in molecular farming- a brief overview. Plant Biotech J 2010;8:525-528
3. Twyman RM, Schillberg S, Fischer R. The Production of Vaccines and Therapeutic Antibodies in Plants. In: Wang A, Ma S (ed). Molecular Farming in Plants: Recent Advances and Future Prospects. Springer Science Business Media BV, 2012,145-159
4. Gupta SK, Shukla P. Microbial platform technology for recombinant antibody fragment production: A review. Crit Rev Microbiol 2016;15:1-12
5. Horn ME, Woodard SL, Howard JA. Plant molecular farming: systems and products. Plant Cell Rep 2004;22:711–720. doi:10.1007/s00299-004-0767-1
6. Hefferon KL. The Mucosal Immune Response to Plant-Derived Vaccines. Pharm Res 2010;27:2040–2042. doi:10.1007/s11095-010-0168-9

7. Wujek P, Kida E, Walus M et al. N-glycosylation is crucial for folding, trafficking and stability of human tripeptidyl-peptidase. J BiolChem 2004;279(13):12827-12829

8. Castilho A, Steinkeliner H. Glyco-engineering in plants to produce human-like N-glycan structures. Biotechnol J 2012;7:1088-1098

9. Malembic S, Saillard C, Bové JM et al. Effect of polyclonal, monoclonal and recombinant single-chain variable fragment antibodies on *in vitro* morphology, growth, and metabolism of the phytopathogenic mollicute, *Spiroplasma citri*. Appl Environ Microbiol 2002;68:2113-2119

10. Peschen D, Li HP, Fischer R et al. Fusion proteins comprising a Fusarium-specific antibody linked to anti-fungal peptides protects plants against fungal pathogens. Nat Biotechnol 2004;22(6):732-738

11. Rybicki EP. Plant-made vaccines for humans and animals. Plant Bitoechnol J 2010;8:620-637

12. Grill LK, Kenneth EP, Gregory PP. Use of plant viruses for production of plant-derived vaccines. Crit Rev Plant Sci 2005;24:309-323

13. Obembe OO, Popoola JO, Leelavathi S, Reddy SV (2011) Advances in plant molecular farming. BiotechnolAdv 29 (2):210-222

14. Yusibov V, Streatfield SJ, Kushnir N et al. Hybrid Viral Vectors for Vaccine and Antibody Production in Plants. Curr Pharm Des 2013;19(31):5574-5586

15. Singh PK, Shukla P. Systems biology as an approach for deciphering microbial interactions. Brief Funct Genomics 2015;142:166-168. doi:10.1093/bfgp/elu023

16. Imam J, Singh PK, Shukla P. Plant microbe interactions in post genomic era: perspectives and applications. Front Microbiol 2016;7:1488. doi:10.3389/fmicb.2016.01488

17. Laere E, Ling APK, Wong YP et al. Plant-Based Vaccines: Production and Challenges. J Bot 2016;2016:Article ID 4928637. doi:10.1155/2016/4928637

18. Curtiss R, Cardineau GA. Oral immunization by transgenic plants. Patent No. 5,654,184 A. U.S. Patent and Trademark Office, Washington DC, 1997.

19. Rosales-Mendoza S, Soria-Guerra RE, Moreno-Fierros L et al. Immunogenicity of nuclear-encoded LTB:ST fusion protein from *Escherichia coli* expressed in tobacco plants. Plant Cell Rep 2011;30(6):1145-52. doi:10.1007/s00299-011-1023-0

20. Daniell H, Lee SB, Panchal T et al. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol 2001;311(5):1001-9. doi:10.1006/jmbi.2001.4921

21. Chikwamba R, Cunnick J, Hathaway D et al. A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin LT and cholera toxin CT. Transgenic Res 2002;11:479-493

22. Soh HS, Chung HY, Lee HH, et al. Expression and functional validation of heat-labile enterotoxin B (LTB) and cholera toxin B (CTB) subunits in transgenic rice (*Oryzasativa*). Springer Plus 2015;4:148. doi:10.1186/s40064-015-0847-4.

23. Cañizares MC, Nicholson L, Lomonossoff P. Use of viral vectors for vaccine production in plants. Immunol Cell Biol 2005;83:263-270

24. Haan L, Hirst TR. Cholera toxin: A paradigm for multi-functional engagement of cellular mechanisms (Review). Mol Membr Biol 2004;21:77-92

25. Mikschofsky H, König P, Keil GM et al. Cholera toxin B (CTB) is functional as an adjuvant for cytoplasmatic proteins if directed to the endoplasmatic reticulum (ER), but

not to the cytoplasm of plants. Plant Sci 2009;177(1):35–42. doi:10.1016/j.plantsci.2009.03.010

26. Lomonossoff GP, Johnson JE. Use of macromolecular assemblies as expression systems for peptides and synthetic vaccines. Curr Opin Struct Biol 1996;6(2):176-182

27. Zhang X, Buehner NA, Hutson AM et al. Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. Plant Biotechnol J 2006;4:419–432. doi:10.1111/j.1467-7652.2006.00191.x

28. Liu L, Cañizares MC, Monger W et al. Cowpea mosaic virus-based systems for the production of antigens and antibodies in plants. Vaccine 2005;23(15):1788-92.

29. Portaa C, Spalla VE, Findlaya KC et al. Cowpea mosaic virus-based chimaeras: Effects of inserted peptides on the phenotype, host range, and transmissibility of the modified viruses. Virology 2003;310(1):50–63. doi:10.1016/S0042-6822(03)00140-5

30. Herbst-Kralovetz M, Mason HS, Chen Q. Norwalk virus-like particles as vaccines.Expert Rev Vaccines 2010;9(3):299–307. doi:10.1586/erv.09.163

31. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci U S A 1992;89(24):11745-9.

32. Richter LJ, Thanavala Y, Arntzen CJ et al. Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nature Biotechnol 2000;18:1167-1171. doi:10.1038/81153

33. Lou X-M, Yao Q-H, Zhang Z et al. Expression of the Human Hepatitis B Virus Large Surface Antigen Gene in Transgenic Tomato Plants. Clin Vaccine Immunol 2007;14(4):464-469. doi:10.1128/CVI.00321-06

34. Ortega-Berlanga B, Musiychuk K, Shoji Y et al. Engineering and expression of a RhoA peptide against respiratory syncytial virus infection in plants. Planta 2016;243(2):451-8.

35. Cruz SS, Chapman S, Roberts AG et al. Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl Acad Sci USA 1996;93:6286-6290

36. O'Brien GJ, Bryant CJ, Voogd C et al. Rotavirus VP6 Expressed by PVX Vectors in *Nicotiana benthamiana* Coats PVX Rods and Also Assembles into Virus like Particles. Virology 2000;270(2):444-453

37. Marconi G, Albertini E, Barone P et al. In planta production of two peptides of the Classical Swine Fever Virus CSFV E2 glycoprotein fused to coat protein of potato virus X. BMC Biotechnol 2006;6:29

38. Zelada AM, Calamante G, de la Paz Santangelo M et al. Expression of tuberculosis antigen ESAT-6 in *Nicotiana tabacum* using a potato virus X-based vector. Tuberculosis 2006;86:263-267

39. Uhde-Holzem K, Schlösser V, Viazov S et al. Immunogenic properties of chimeric potato virus X particles displaying the hepatitis C virus hypervariable region I peptide R9. J Virol Methods 2010;166(1-2):12-20

40. Sempere RN, Gómez P, Truniger V et al. Development of expression vectors based on pepino mosaic virus. Plant Methods 2011;7:6. doi:10.1186/1746-4811-7-6

41. Minato N, Komatsu K, Maejima K et al. Efficient foreign gene expression in planta using a *plantago asiatica* mosaic virus-based vector achieved by the strong RNA-silencing suppressor activity of TGBp1. Arch Virol 2014;159(5):885-896

42. Mechtcheriakova IA, Eldarov MA, Nicholson L et al. The use of viral vectors to produce hepatitis B virus core particles in plants. J Virol Methods 2006;131(1):10-15

43. Monger W, Alamillo JM, Sola I et al. An antibody derivative expressed from viral vectors passively immunizes pigs against transmissible gastroenteritis virus infection when supplied orally in crude plant extracts. Plant Biotechnol J 2006;4(6):623-631

44. Zhang C, Bradshaw JD, Whitham SA et al. The Development of an Efficient Multipurpose Bean Pod Mottle Virus Viral Vector Set for Foreign Gene Expression and RNA Silencing. Plant Physiol 2010;153(1):52-65

45. Marsian J, Fox H, Bahar MW et al. Plant-made polio type 3 stabilized VLPs-a candidate synthetic polio vaccine. Nat Commun 2017;8(1):245. doi: 10.1038/s41467-017-00090-w.

46. Elder K, Cohn J. Vaccines in Developing Countries: Why the High Prices? http://blogs.plos.org/speakingofmedicine/2013/04/23/vaccines-in-developing-countries-why-the-high-prices (24 Feb 2017, date last accessed).

47. Mason HS, Warzecha H, Mor T et al. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol Med 2002;8: 324–329. doi: 10.1016/S1471-4914(02)02360-2

48. Zelenyánszki H, Mezei Z, Hamar E et al. The green capsule: edible vaccine production in transgenic plants. New Biotechnology 2016;33:S76. doi:10.1016/j.nbt.2016.06.980

49. Fischer R, Schillberg S. Molecular Farming. Encyclopaedia of Applied Plant Sciences 2017;2:77-82. doi:10.1016/B978-0-12-394807-6.00159-3

50. MacDonald J, Doshi K, Dussault M et al. Bringing plant-based veterinary vaccines to market: Managing regulatory and commercial hurdles Biotechnol Adv 2015;33(8):1572-1581

51. Makvandi-Nejad S, McLean MD, Hirama T et al. Transgenic tobacco plants expressing a dimeric single-chain variable fragment scfv antibody against *Salmonella enterica* serotype Paratyphi B. Transgenic Res 2005;14(5):785-792

52. Lai H, He J, Hurtado J et al. Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single□chain variant produced in glycoengineered plants. Plant biotechnol 2014;12(8):1098-107

53. Rosenberg Y, Sack M, Montefiori D et al. Rapid high-level production of functional HIV broadly neutralizing monoclonal antibodies in transient plant expression systems. PLoS One 2013;8(3):e58724.

54. Sainsbury F, Lavoie PO, D'Aoust MA et al. Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. Plant Biotechnol J 2008;6:82-92

55. Yu J, Langridge WH. A plant-based multicomponent vaccine protects mice from enteric diseases. Nat Biotechnol 2001;19(6):548-52. doi: 10.1038/89297

56. Rosales-Mendoza S, Sández-Robledo C, Bañuelos-Hernández B et al. Corn-based vaccines: current status and prospects. Planta 2017;245(5):875-888. doi: 10.1007/s00425-017-2680-1

57. Rosales-Mendoza S, Salazar-González JA, Decker EL et al. Implications of plant glycans in the development of innovative vaccines. Expert Rev Vaccines 2016;15:915-925. doi:10.1586/14760584.2016.1155987

58. Zhang M, Viennois E, Prasad M et al. Edible ginger-derived nanoparticles: A novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer. Biomaterials 2016;101:321-340

59. Arakawa T, Yu J, Chong DK et al. A plant -based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. Nat Biotechnol 1998;16(10):934-938. doi:10.1038/nbt1098-934

60. Daniell H, Singh ND, Mason H et al. Plant-made vaccine antigens and Biopharmaceuticals. Trends Plant Sci 2009;14:669–679.

61. Daniell H. Transgene containment by maternal inheritance: effective or elusive? Proc Natl Acad Sci USA 2007;104:6879–6880

62. Bock R, Warzecha H. Solar-powered factories for new vaccines and antibiotics. Trends Biotechnol 2010;28:246–252

63. Ruhlman T, Verma D, Samson N et al. The role of heterologous chloroplast sequence elements in transgene integration and expression. Plant Physiol 2010;152:2088–2104

64. Lössl AG, Waheed MT. Chloroplast-derived vaccines against human diseases: achievements, challenges and scopes. Plant Biotechnology Journal 2011;9(5):527–539

65. Adem M, Beyene D, Feyissa T. Recent achievements obtained by chloroplast transformation. Plant Methods 2017;13:30. doi:10.1186/s13007-017-0179-1

66. Cardi T, Lenzi P, Maliga P. Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev Vaccines 2010;9:893–911

67. Fernandez-San Millan A, Ortigosa SM, Hervas-Stubbs S et al. Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. Plant Biotechnol J 2008;6:427–441

68. Morgenfeld M, Segretin ME, Wirth S et al. Potato virus X coat protein fusion to Human Papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. Mol Biotechnol 2009;43:243–249

69. Zhou YX, Lee MY, Ng JM et al. A truncated hepatitis E virus ORF2 protein expressed in tobacco plastids is immunogenic in mice. World J Gastroenterol 2006;12:306–312

70. Birch-Machin I, Newell CA, Hibberd JM et al. Accumulation of rotavirus VP6 protein in chloroplasts of transplastomic tobacco is limited by protein stability. Plant Biotechnol J 2004;2:261–270

71. Zhou F, Badillo-Corona JA, Karcher D et al. High-level expression of human immunodeficiency virus antigens from the tobacco and tomato plastid genomes. Plant Biotechnol J 2008;6:897–913

72. Dreesen IA, Hamri GC, Fussenegger M. Heat-stable oral alga-based vaccine protects mice from Staphylococcus aureus infection. J Biotechnol 2010;145:273–280

73. Ruhlman T, Ahangari R, Devine A et al. Expression of cholera toxin B-proinsulin fusion protein in lettuce and tobacco chloroplasts–oral administration protects against development of insulitis in non-obese diabetic mice. Plant Biotechnol J 2007;5:495–510

74. Tissot G, Canard H, Nadai M et al. Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnol J 2008;6:309–320

75. Ruiz ON, Daniell H. Engineering cytoplasmic male sterility via the chloroplast genome by expression of ß-ketothiolase. Plant Physiol 2005;138:1232–1246

76. Waheed MT, Ismail H, Gottschamel J et al. Plastids: The Green Frontiers for Vaccine Production. Front Plant Sci 2015;6:1005. doi:10.3389/fpls.2015.01005

77. Bortesia L, Fischera R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 2015;33(1):41-52. doi.:10.1016/j.biotechadv.2014.12.006

78. Gaj T, Sirk SJ, Shui S et al. Genome-Editing Technologies: Principles and Applications. Cold Spring Harb Perspect Biol 2016;8:a023754. doi: 10.1101/cshperspect.a023754

79. Basu S, Rabara RC, Negi S et al. Engineering PGPMOs through Gene Editing and Systems Biology: A Solution for Phytoremediation? Trends Biotechnol 2018. doi: 10.1016/j.tibtech.2018.01.011.

80. Gupta SK, Shukla P. Gene editing for cell engineering: trends and applications. Crit Rev Biotechnol 2017;37(5):672-84. doi: 10.1080/07388551.2016.1214557

81. Hou Z, Zhang Y, Propson NE et al. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. Proc Natl Acad Sci 2013;110:15644–15649

82. Mali P, Aach J, Stranges PB et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 2013;31:833–838

83. Ran FA, Hsu PD, Lin CY et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 2013;154:1380–1389

84. Torres R, Martin MC, Garcia A et al. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat Commun 2014;5:3964

85. Baltes NJ, Voytas DF. Enabling plant synthetic biology through genome engineering. Trends Biotechnol 2015;33:120–131

86. Wang Y, Cheng X, Shan Q et al. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 2014;32:947–951

87. Haun W, Coffman A, Clasen BM et al. Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J 2014;12:934–940

88. Lawrenson T, Shorinola O, Stacey N et al. Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. Genome Biol 2015;16:258. doi.:10.1186/s13059-015-0826-7

89. Čermák T, Baltes NJ, Čegan R et al. High-frequency, precise modification of the tomato genome. Genome Biol 2015;16:232

90. Wang ZP, Xing HL, Dong L et al. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol 2015;16:144. doi.: 10.1186/s13059-015-0715-0

91. Kawakatsu T, Kawahara Y, Itoh T et al. A Whole-Genome Analysis of a Transgenic Rice Seed-Based Edible Vaccine Against Cedar Pollen Allergy. DNA Res 2013;20(6):623–631. doi: 10.1093/dnares/dst036

92. Nielsen M, Lundegaard L, Worning P et al. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci 2003;12(5):1007-1017

93. Bhasin M, Raghava GPS. A hybrid approach for predicting promiscuous MHC class I restricted T cell epitopes. J Biosci 2007;32: 31-42. doi:10.1007/s12038-007-0004-5

94. Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO et al. An overview of bioinformatics tools for epitope prediction: implications on vaccine development. Journal Biomed Inform 2015;53:405-14.

95. Flower DR. Towards in-silico prediction of immunogenic epitopes. Trends Immunol 2003;2412:667-674

96. De Jaeger G, De Wilde C, Eeckhout D et al. The plantibody approach: expression of antibody genes in plants to modulate plant metabolism or to obtain pathogen resistance. Plant Mol Biol 2000;43(4):419-28

97. Dobhal S, Chaudhary VK, Singh A et al. Expression of recombinant antibody (single chain antibody fragment) in transgenic plant *Nicotiana tabacum* cv. Xanthi. Mol Biol Rep 2013;40(12):7027-37. doi: 10.1007/s11033-013-2822-x

98. Sabalza M, Christou, Capell T. Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks. Biotechnol Lett 2014;36(12):2367–2379. doi:10.1007/s10529-014-1621-3

99. Ghannam A, Kumari S, Muyldermans S et al. Camelid nanobodies with high affinity for broad bean mottle virus: a possible promising tool to immunomodulate plant resistance against viruses. Plant Mol Biol 2015;87(4-5):355-69.

100. Falk LA, Ball LK. Current status and future trends in vaccine regulation — USA. Vaccine 2001;19(13–14):1567-1572. doi:10.1016/S0264-410X(00)00353-4

101. World Health Organization Report (2005) WHO Informal Consultation on Scientific Basis for Regulatory Evaluation of Candidate Human Vaccines from Plants, WHO Quality Assurance and Safety of Biologicals. WHO, Geneva, Switzerland

102. Arango IP, Rubio EL, Anaya ER et al. Expression of the rabies virus nucleoprotein in plants at high-levels and evaluation of immune responses in mice. Plant Cell Rep 2008;27(4): 677–685

103. Sharma M, Sood B. A banana or a syringe: journey to edible vaccines. World J Microbiol Biotechnol 2011;27(3):471–477

104. Kanagaraj AP, Verma D, Daniell H. Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts. Plant Mol Biol 2011;76(3–5):323–333.

105. Redkiewicz P, Sirko A, Kanem KA, Góra-Sochacka A. Plant expression systems for production of hemagglutinin as a vaccine against influenza virus. Acta Biochimica Polonica 2014;61:551–560.

106. Joung YH, Youm JW, Jeon JH et al. Expression of the hepatitis B surface S and preS2 antigens in tubers of *Solanumtu berosum*. Plant Cell Rep 2004;22(12):925-30.

107. Li T, Sun JK, Lu ZH et al. Transformation of HBsAg (hepatitis B surface antigen) gene into tomato mediated by *Agrobacterium tumefaciens*. Czech J Genet Plant Breed 2011;47:69-77

108. Zungu, N, Lotter T, Dube N et al. Expression of Rabies antibodies in tobacco and maize. SASBMB Conference, Grahamstown, 2008. 23-25 January 2008, pp 1

109. Hensel G, Floss DM, Arcalis E et al. Transgenic Production of an Anti HIV Antibody in the Barley Endosperm. PLoS ONE 2015;10(10):e0140476. doi:10.1371/journal.pone.0140476

110. Marquet-Blouin E, Bouche FB, Steinmetz A et al. Neutralizing immunogenicity of transgenic carrot *Daucus carota* L.-derived measles virus hemagglutinin. Plant Mol Biol 2003;51(4):459-469

111. Rademacher T, Sack M, Arcalis E et al. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV□1 and contains predominantly single□GlcNAc N□glycans. Plant Biotechnol J 2008;6(2):189-201

112. Jani D, Meena LS, Rizwan-ul-Haq QM et al. Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res 2002;11(5):447-454

113. Wu YZ, Li JT, Mou ZR et al. Oral immunization with rotavirus VP7 expressed in transgenic potatoes induced high titers of mucosal neutralizing IgA. Virology 2003;313(2):337-342

114. Rigano MM, Alvarez ML, Pinkhasov J et al. Production of a fusion protein consisting of the enterotoxigenic *Escherichia coli* heat-labile toxin B subunit and a tuberculosis antigen in Arabidopsis thaliana. Plant Cell Rep 2004;22:502-508. doi:10.1007/s00299-003-0718-2

115. Phoolcharoen W, Bhoo SH, Lai H et al. Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*. Plant Biotechnol J 2011;9(7):807–816. doi:10.1111/j.1467-7652.2011.00593.x

116. Pan L, Zhang Y, Wang Y et al. Foliar extracts from transgenic tomato plants expressing the structural polyprotein, P1-2A, and protease, 3C, from foot-and-mouth disease virus elicit a protective response in guinea pigs. Vet Immunol Immunopathol 2008;121:83–90.

117. Li Y, Sun M, Liu J et al. High expression of foot-and-mouth disease virus structural protein VP1 in tobacco chloroplasts. Plant Cell Rep 2006;25:329–333.

118. Jones RM, Chichester JA, Mett V et al. A Plant-Produced Pfs25 VLP Malaria Vaccine Candidate Induces Persistent Transmission Blocking Antibodies against *Plasmodiumfalciparum* in Immunized Mice. PLoS ONE 2013;8(11):e79538. doi:10.1371/journal.pone.0079538

119. Haq TA, Mason HS, Clements JD et al. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 1995;268:714-716

120. Drake PM, Szeto TH, Paul MJ et al. Recombinant biologic products versus nutraceuticals from plants–a regulatory choice? Brit J Clin Pharmaco 2017;83(1):82-7

121. Tacket CO, Mason HS, Losonsky G et al. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 1998;4(5):607-609

122. Merlin M, Pezzotti M, Avesani L. Edible plants for oral delivery of biopharmaceuticals. Brit J Clin Pharmaco 2017;83(1):71-81.

123. Lai H, Chen Q. Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. Plant Cell Rep 2012;31(3):573–584

124. Su J, Sherman A, Doerfler PA et al. Oral delivery of Acid Alpha Glucosidase epitopes expressed in plant chloroplasts suppresses antibody formation in treatment of Pompe mice. Plant Biotechnol J 2015;13(8):1023-32

125. Kapusta J, Modelska A, Figlerowicz M et al. A plant-derived edible vaccine against hepatitis B virus. FASEB J 1999;13:1796-1799

126. Takeyama N, Kiyono H, Yuki Y. Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. Ther Adv vaccines 2015;3(5-6):139-54

127. Porta C, Spall VE, Loveland JE et al. Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. Virology 1994;202:949-955

128. McLain L, Durrani Z, Wisniewski LA et al. Stimulation of neutralizing antibodies to human immunodeficiency virus type 1 in three strains of mice immunized with a 22-mer amino acid peptide expressed on the surface of a plant virus. Vaccine 1996;14(8):799-810

129. Modelska A, Dietzschold B, Sleysh N et al. Immunization against rabies with plant-derived antigen. Proc Natl AcadSci USA 1998;95(5):2481-2485

130. Dalsgaard K, Uttenthal A, Jones TD et al. Plant derived vaccine protects target animalsagainst a viral disease. Nat Biotechnol 1997;15:248-252

131. Brennan FR, Bellaby T, Helliwell SM et al. Chimeric Plant Virus Particles Administered Nasally or Orally Induce Systemic and Mucosal Immune Responses in Mice. J Virol 1999;732:930-938

132. Lefranc MP, Clement O, Kaas Q et al. IMGT-Choreography for Immunogenetics and Immunoinformatics. In Silico Biol 2005;5(1):45–60

133. Rammensee H, Bachmann J, Emmerich NP. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;503-4:213-219

134. Peters B, Sidney J, Bourne P et al. The Immune Epitope Database and Analysis Resource: From Vision to Blueprint. PLoSBiol 2005;33:e91

135. Sainsbury F, Lavoie PO, D'Aoust MA et al. Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. Plant Biotechnol J 2008;6:82-92

136. Bhasin M, Singh H, Raghava G. MHCBN: a comprehensive database of MHC binding and non-binding peptides. Bioinformatics 2003;195:665-666

137. Robinson J, Waller MJ, Stoehr P et al. IPD-the Immuno Polymorphism Database. Nucleic Acids Res 2005;33:D523-D526

138. Schlessinger A, Ofran Y, Yachdav G et al. Epitome: database of structure-inferred antigenic epitopes. Nucleic Acids Res 2006;34:D777-780

139. Allergen Nomenclature, WHO/IUIS Allergen Nomenclature Sub-Committee. http://www.allergen.org/. 05 Feb 2018, date last accessed).

140. Gendel SM. Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. Adv Food Nutr Res 1998;42:63-92

141. Ivanciuc O, Schein CH, Braun W. SDAP: database and computational tools for allergenic proteins. Nucleic Acids Res 2003;31:359-362

142. Hileman RE, Silvanovich A, Goodman RE et al. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. Int Arch Allergy Immunol 2002;128(4):280-291

143. Mari A, Mari V, Ronconi A. Allergome - a database of Allergenic molecules: structure and data implementations of a web-based resource. J Allergy ClinImmunol 2005;115:S87

144. National Institute of allergy and infectious diseases. https://www.niaid.nih.gov/. (05 Feb 2018, date last accessed).

145. Ansari HR, Flower DR, Raghava GPS. AntigenDB: an immunoinformatics database of pathogen antigens. Nucleic Acids Res 2010;38: D847-853. doi:10.1093/nar/gkp830

146. Saha S, Bhasin M, Raghava GP. Bcipep: a database of B-cell epitopes. BMC Genomics 2005;6:79

147. Hundal J, Carreno BM, Petti AA et al. pVAC-Seq: A genome-guided in silico approach to identifying tumorneoantigens. Genome Med 2016;8(1):11. doi:10.1186/s13073-016-0264-5

148. Wang M, Lamberth K, Harndahl M et al. CTL epitopes for influenza A including the H5N1 bird flu; genome-,pathogen-, and HLA-wide screening. Vaccine 2007;25:2823–2831. doi:10.1016/j.vaccine.2006.12.038

149. Cheung YK, Cheng SCS, Ke Y et al. Two novel HLA-A*0201 T-cell epitopes in avian H5N1 viral nucleoprotein induced specific immune responses in HHD mice. Vet Res 2010;41(2):24. doi:10.1051/vetres/2009071

**Key Points**

1. Plant expression systems have several intrinsic advantages in terms of speed, costs, scalability and safety.
2. Plant based antigen expression has emerged as a viable technology for the production of recombinant proteins.
3. To date, numerous antibodies are reported in a variety of different plant species
4. The success of plant derived antibodies mainly depends on the development of transient and stable transformation for commonly grown crops, and ease of cultivation.

**Figure legends:**

**Figure 1.** Demonstration of approaches used for the production of plantibodies. In silico tools assist in epitope prediction for antibodies to be expressed as plant derived antibodies (PDAbs). The gene designed for vaccination is integrated into a vector and transferred into plant cells via suitable gene transfer approaches.

**Figure 2.** Recombination and selective breeding approaches used for the production of PDAbs

**Figure 3.** Schematic overview of 2A function. **(A)** The foot-and-mouth disease virus polyprotein includes the L proteinase ($L^{pro}$), the capsid proteins domain, 2A and two further domains comprising the replicative proteins. The 18aa long 2A sequence is shown together with the site of cleavage (arrow) and the N-terminal proline of 2B, instantly downstream of 2A. **(B)** Gene sequences 1 (with no stop codon) and 2 are concatenated into a single (trans) gene *via* a 2A linker. The translation products are synthesised in an equimolar ratio, while, protein 1 upstream of 2A bears a C-terminal extension of 2A, and protein 2 bears an N-terminal proline residue

**Figure 4.** Flow-chart representing algorithms for the implementation of epitope-based vaccine design.

**Key Points**

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4. The success of plant derived antibodies mainly depends on the development of transient and stable transformation for commonly grown crops, and ease of cultivation.

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**Table 1.** Transgenic/ transient expression of different antigenic epitopes as vaccine candidates in various plant systems

| Plant expression system | Method of transgenesis | Pathogen | Antigenic epitope | Reference |
|---|---|---|---|---|
| *Nicotiana benthamiana* | *Agrobacterium* mediated transformation | Infleunza | Hemagglutinin (HA) | [105] |
| Potato | *Agrobacterium* mediated transformation | Hepatitis B virus | hepatitis B surface antigen (HBsAg) surface S and preS2 antigen | [106] |
| Tomato | *Agrobacterium* mediated transformation | Hepatitis B virus | hepatitis B surface antigen (HBsAg) | [107] |
| Tobacco and maize | *Agrobacterium* mediated transformation | Rabies | Monoclonal antibody (MAb) E559 | [108] |
| Barley | *Agrobacterium tumefaciens* mediated | HIV | Anti-HIV-1 monoclonal antibody 2G12 | [109] |
| Carrot | *Agrobacterium* mediated transformation | *E. coli* | LT-B | [110] |
| Maize | Particle bombardment | *Vibrio cholera* *E. coli* | Cholera toxin beta subunit (CTB) and LT-B | [21] |
| Maize endosperm | Particle bombardment | HIV | Anti HIV 2G12 | [111] |
| Tobacco | *Agrobacterium*-mediated transformation | *Vibrio cholera* | cholera toxin subunit B (CTB) | [112] |
| Potato | *Agrobacterium*-mediated transformation | Rota virus | Rota Virus VP7 | [113] |
| *Arabidopsis thaliana* | *Agrobacterium*-mediated transformation | *Mycobacterium tuberculosis* | Tuberculosis antigen | [114] |
| *Nicotiana benthamiana* | Agroinfiltration | Ebola virus | Ebola glycoprotein (GP1) | [115] |
| Tomato | *Agrobacterium*- | Foot-and-mouth | P1-2A, and | [116] |

| | | | | |
|---|---|---|---|---|
| | mediated transformation | disease virus | protease, 3C | |
| Tobacco | Biolistic method | Foot-and-mouth disease virus | VP1 | [117] |
| *Nicotiana benthamiana* | *Agrobacterium*-mediated transformation | *Plasmodium falciparum* | Pfs25-CP VLP | [118] |

**Table 2.** Immunological responses of vaccines against animals/human pathogens produced through genomic transformation of plants with different antigenic gene epitopes

| Pathogen | Antigen | Plant Expression System | Test system: Immune response | Reference |
|---|---|---|---|---|
| Enterotoxigenic *E.coli* | Enterotoxin B subunit (LT-B) | Tobacco leaf | Mouse:Serum and mucosal antibodies to LT-B | [119] [120] |
| Enterotoxigenic *E. coli* | Enterotoxin B subunit (LT-B) | Potato tuber | Mouse:Serum and mucosal antibodies to LT-B; protected against challenge with toxin | [121] [122] |
| Norwalk virus | Capsidprotein | *Nicotiana benthamiana* | | [123] |
| *Vibrio cholerae* | Cholaratoxin B subunit (CT-B) | Potato tuber and leaf callus | Mouse:Serum antibodies to CT-B with tuber or callus; decreased severity | [59] [124] |
| Hepatitis B virus | Suface antigen (HbsAg) | Lupin callus and Lettuce leaf | Mouse:Serum antibodies to (HbsAg) Human:Serum antibodies to HbsAg | [125] [126] |
| Tuberculosis | Tuberculosis antigen | *Arabidopsis thaliana* | | [114] |

**Table 3.** Immunological response of transiently expressed vaccines against animals/human pathogens in different plant systems

| Pathogen | Antigenic epitope | Plant | Source of viral coat protein | Test system: Immune response | Reference |
|---|---|---|---|---|---|
| Human rhinovirus | VP1 | Cowpea leaf | CPMV | Rabbit:serum antibodies to VP1 | [127] |
| Human immune Deficiency Virus (HIV-1) | Gp41 | Cowpea leaf | CPMV | Mouse:serum antibodies to gp41 | [128] |
| Rabies virus | Drg 24 | Tobacco leaf and spinach leaf | AMV | Mouse:serum antibodies and mucosal antibodies to Drg24 | [129] |
| Mink entritis virus | Capsid proteins VP2 | Black eyed bean leaf | CPMV | Mouse:serum antibodies to VP2 | [130] |
| *Staphylococcus aureus* | D2 Peptide of FnBP Binding protein | Cowpea leaf | CPMV | Mouse:serum antibodies to D2 | [131] |
| Measles virus | Hemagglutinin (H) glycoprotein | Carrot | AMV | Mouse:serum antibodies to H | [110] |

**Table 4.** An overview of various *In Silico* methods of Epitope prediction in Plantibodies engineering

| Database and its URL | Brief description | Functions | Remarks | Reference |
|---|---|---|---|---|
| IMGT (International ImMunoGeneTics information system) http://www.imgt.org/ | It is immunogenetics and immunoinformatics resource | provides a common access to sequence, genome and structure Immunogenetics data, based on the concepts of IMGT-ONTOLOGY | Created by Marie-PauleLefranc (Université de Montpellier and CNRS)., Year: 1989 | [132] |
| SYFPEITHI (A Database for MHC Ligands and Peptide Motifs) http://www.syfpeithi.de/ | This comprises more than 7000 peptide sequences known to bind class I and class II MHC molecules. | The prediction is based on published motifs (pool sequencing, natural ligands) and takes into consideration the amino acids in the anchor and auxiliary anchor positions, as well as other frequent amino acids. | | [133] |
| IEDB (Immune Epitope Database and Analysis Resource) http://www.immuneepitope.org/ | It is a resource center for data related to antibody and T cell epitopes for humans, non-human primates, rodents, and other animal species. | It predicts antibody and T cell epitopes has epitope analysis tools as well as peptide processing predictions | As on April 26, 2015 it has<br>Peptidic Epitopes-130671,<br>Non-Peptidic Epitopes-2199<br>T Cell Assays-271157<br>B Cell Assays - 180401<br>MHC Ligand Assays-323330<br>Epitope Source Organisms-3383<br>Restricting MHC Alleles-683<br>References-16860 | [134] |
| AntiJen http://www.jenner.ac.u | It contains entries of binding data on MHC ligands, | It accepts epitope strings with variable amino acid positions and alternate | contains over 24,000 entries of binding data on MHC ligands, | [135] |

| | | | | |
|---|---|---|---|---|
| k/antijen/ | TR-peptide-MHC complexes, T cell epitopes, TAP, B cell epitopes and immunological protein–protein interactions. | amino acid in any epitope string, an optional filter can be used to target experimental data of interest, delimiting it also searches on the basis of epitope length | Peptide library, copy numbers and diffusion coefficient data are also included | |
| MHCBN (Major Histocompatibility Complex (MHC) Binding, Non-binding peptides and T-cell epitopes) http://www.imtech.res.in/raghava/mhcbn/ | It contains data of MHC binders and non-binders, TAP binders and non-binders, and T-cell epitopes | The database provides information about allele specific MHC binding peptides, MHC non-binding, TAP binding, TAP non-binding peptides and T-cell epitopes reported in literature | 25860 MHC binders and non-binders, 1053 TAP binders and non-binders, and 6722 T cell epitopes | [136] |
| IPD (Immuno Polymorphism Database)http://www.ebi.ac.uk/ipd/ | IPD is a set of specialist databases related to the study of polymorphic genes in the immune system. The IPD-MHC Database provides a centralised repository for sequences of the Major Histocompatibility Complex (MHC) from a number of different species. | It consists of four databases: IPD-KIR for sequences of killer-cell IG-like receptors; IPD-MHC for MHC sequences of different species; IPD-HPA for alloantigens expressed only on platelets and IPD-ESTAB for access to the European Searchable Tumour Cell-Line Database, a cell bank of immunologically characterized melanoma cell lines | Through a number of international collaborations IPD is able to provide the MHC sequences of different species | [137] |
| Epitome http://www.cubic.bioc.columbia.ed | Epitome stores information of 142 antigens fromprotein– | It includes the detailed description of residues involved in the interaction and their | Along with that interactions can be visualized using an interface into Jmol. | [138] |

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|---|---|---|---|---|
| u/services/Epitome/ | antibody complex structures | sequence / structure environments. | | |
| Allergen Nomenclature Database http://www.allergen.org/ | This website is the official site for the systematic allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. | The Allergen Nomenclature Database contains information of allergens and isoallergens developed and maintained by the Allergen Nomenclature sub-committee of the IUIS. | Data submissions are accepted and annotated by the committee members | [139] |
| BIFS (Bioinformatics for Food Safety) http://www.iit.edu/~sgendel/fa.htm | BIFS (Bioinformatics for Food Safety) is maintained by Illinois Institute of Technology | | BIFS contains information on 453 food allergens (64 animals, 389 plants), 645 non-food allergens, and 75 wheat gluten proteins | [140] |
| SDAP (Structural Database of Allergenic Proteins) http://www.fermi.utmb.edu/SDAP/ | SDAP is a Web server that integrates a database of allergenic proteins with various computational tools that can assist structural biology studies related to allergens | SDAP stores information of 887 allergenic proteins. It contains various tools for FAO/WHO allergenicitytests and assessing the IgE-binding potential ofgenetically modified food proteins | SDAP contains information about the allergen name, source, sequence, structure, IgE epitopes and literature references. | [141] |
| FARRP (Food Allergy Research and Resource Program) http://www.farrp.org/ | FARRP was established in 1995 as a cooperative venture between the University of Nebraska and seven founding industry charter members. | FARRP contains 1251 sequences of known and putative allergens derived from scientific literature and public databases | FARRP works with research institutions, governmental authorities, consumer groups, and scientific societies around the globe to improve the safety of food products for consumers with food allergies and sensitivities. | [142] |

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| Allergome http://www.allergome.org/ | The Allergome web site has been designed to supply information on Allergenic Molecules (Allergens). | Allergome emphasizes the annotation of allergens that cause IgE-mediated disease. | The database contains information derived from 5800 selected scientific literatures. | [143] |
| ALPSbase (Autoimmune Lymphoproliferative Syndrome Database) | Autoimmune lymphoproliferative syndrome database | It supports basic and applied research to better understand, treat, and ultimately prevent infectious, immunologic, and allergic diseases | The database emphasize on extensive research portfolio of basic and applied research to prevent, diagnose, and treat infectious diseases such as HIV/AIDS and other sexually transmitted diseases, influenza, tuberculosis, malaria, and illness from potential agents of bioterrorism. | [144] |
| AntigenDB http://www.imtech.res.in/raghava/antigendb | The data on antigens were extracted from the primary literature. Antigens provided are from immunological databases (IEDB, MHCBN, AntiJen, BCIPEP) and other databases (e.g. Swiss-Prot) | An immunoinformatics database of pathogen antigens | Provides Sequence, structure, and other data on pathogen antigens. | [145] |
| BCIpep http://bioinformatics.uams.edu/mirror/bcipep/ | The Bcipep is a collection of immunogenic peptides.The database consists of nearly 555 antigenic peptide with varying immunogenic activity. | Bcipep is a database of experimentally determined linear B-cell epitopes of varying immunogenicity collected from literature and other publicly available databases | The database now contains 2700 entries including 1350 epitopes in the non-redundant datasets. | [146] |
| pVAC-Seq https://githu | personalized Variant Antigens by Cancer | A flexible, streamlined computational workflow | help to evaluate tumor-specific neoepitopes in | [147] |

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| b.com/griffithlab/pVAC-Seq | Sequencing (pVAC-Seq) | for identification of personalized Variant Antigens by Cancer Sequencing (pVAC-Seq) that integrates tumor mutation and expression data (DNA- and RNA-Seq) | a much-reduced time, thereby increasing its applicability for clinical use | |
| NetCTL | Prediction HLA-I restricted cytotoxic T cell epitopes | One hundred and thirty one peptides have affinities for the HLA-I supertypes and only 21 were found to induce T cell responses | | [148] |
| SYFPEITHI | Prediction of HLA binding peptides | Two novel HLA-A *0201 restricted epitopes | | [149] |

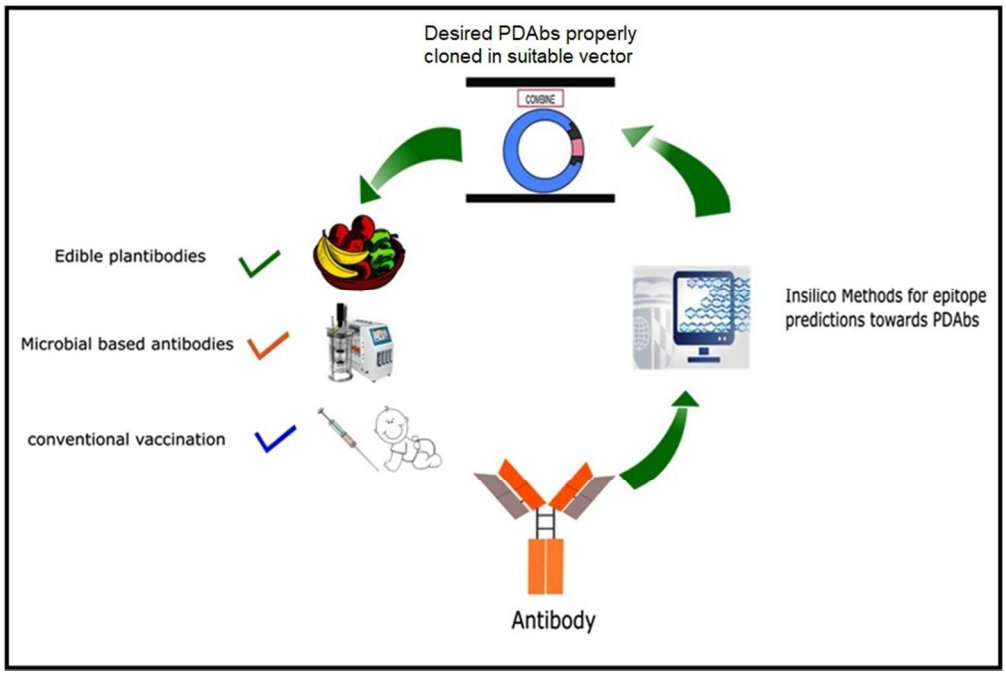


Figure 1: Demonstration of approaches used for the production of plantibodies. In silico tools assist in epitope prediction for antibodies to be expressed as plant derived antibodies (PDAbs). The gene designed for vaccination is integrated into a vector and transferred into plant cells via suitable gene transfer approaches.

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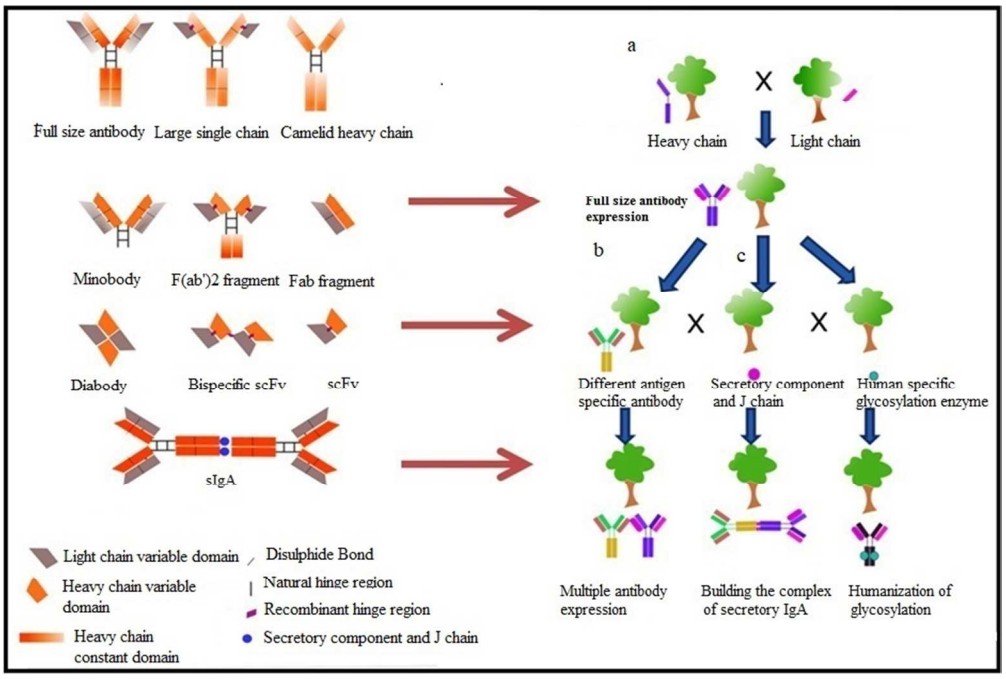


Figure 2. Recombination and selective breeding approaches used for the production of PDAbs

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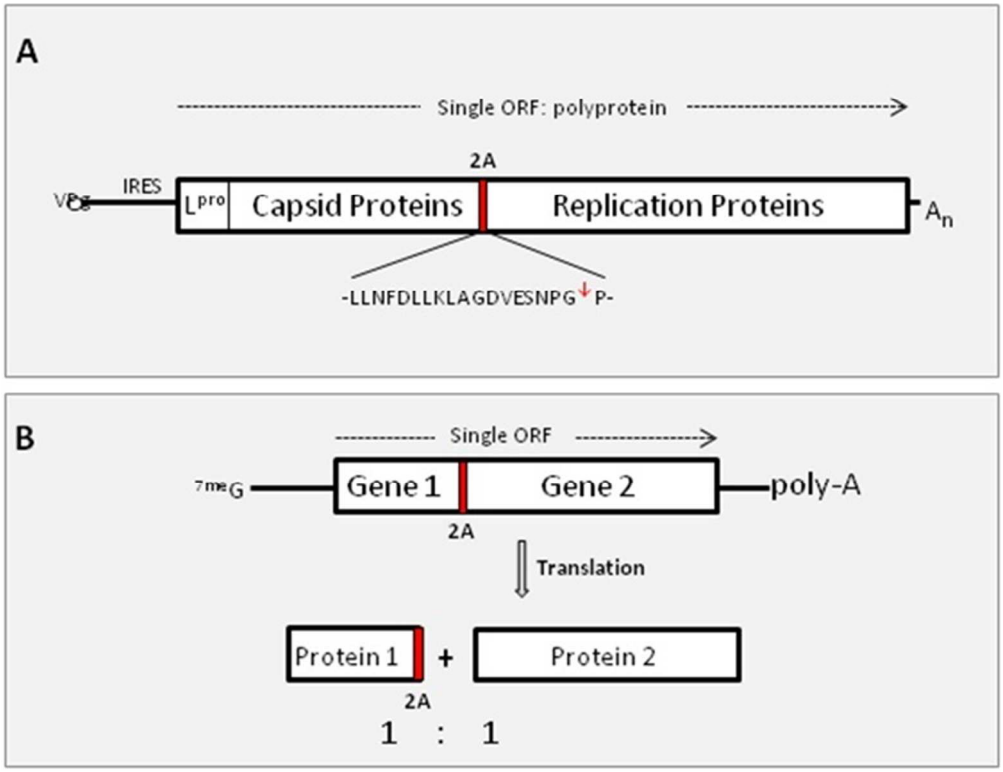


Figure 3. Schematic overview of 2A function. (A) The foot-and-mouth disease virus polyprotein includes the L proteinase (Lpro), the capsid proteins domain, 2A and two further domains comprising the replicative proteins. The 18aa long 2A sequence is shown together with the site of cleavage (arrow) and the N-terminal proline of 2B, instantly downstream of 2A. (B) Gene sequences 1 (with no stop codon) and 2 are concatenated into a single (trans) gene via a 2A linker. The translation products are synthesised in an equimolar ratio, while, protein 1 upstream of 2A bears a C-terminal extension of 2A, and protein 2 bears an N-terminal proline residue

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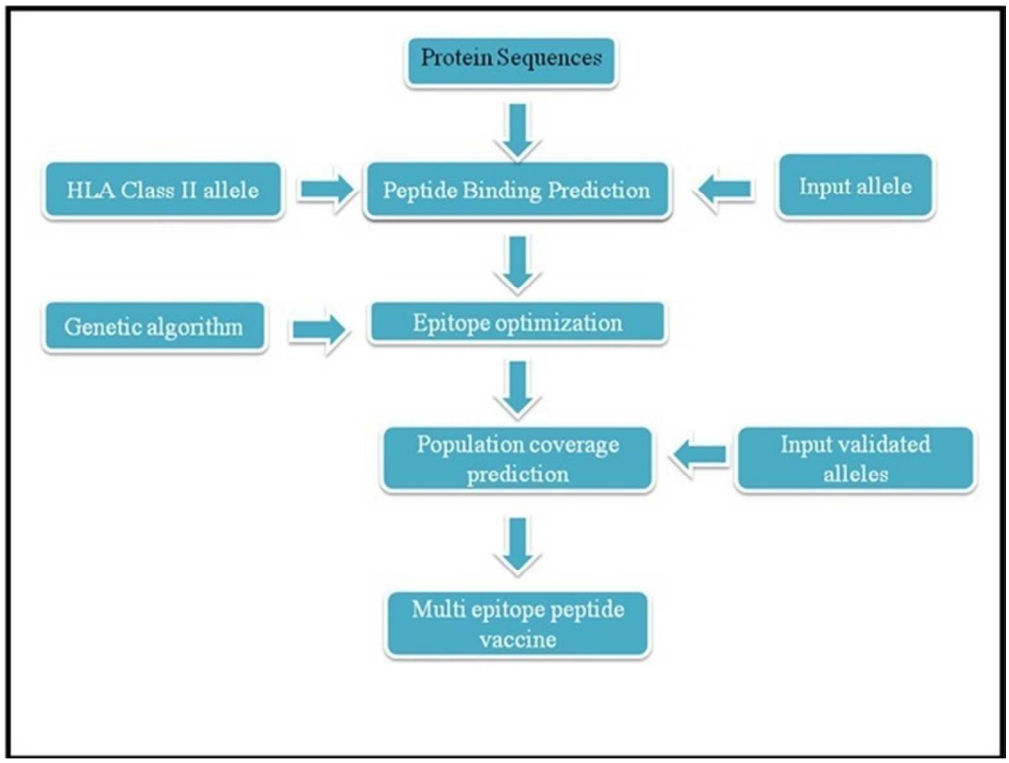


Figure 4. Flow-chart representing algorithms for the implementation of epitope-based vaccine design.

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